Purification of native Sigma class glutathione transferase from *Fasciola hepatica*

Joshua Duncan[1], David Cutress, Russell M. Morphew, Peter M. Brophy*

Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University, Aberystwyth SY23 3DA, UK

ABSTRACT

Fascioliasis is a parasitic disease of grazing livestock and a threat to global food security by significantly reducing the production value of sheep, goats and cattle. Moreover, the zoonotic parasite is also a re-emerging food borne threat to human populations. Driven by climate change, the prevalence of fascioliasis is set to increase. Efforts to control the main causative agent, *Fasciola hepatica*, are hampered by short lived chemotherapy approaches that are becoming increasingly obsolete due to therapeutic failure and resistance. A protective vaccine is urgently needed. A recombinant form of Sigma class glutathione transferase (Hematopoietic Prostaglandin D synthase) from *F. hepatica* (*Fh*GSTS1) with confirmed prostaglandin synthase activity shows immune-modulation activity via suppression of Th17 responses in host dendritic cells. In vaccine trials recombinant *Fh*GSTS1 reduces liver pathology but not worm burden. Native *Fh*GSTS1 is yet to be tested for immune-modulation activities or for vaccine potential, primarily due to the technical difficulty in purifying *Fh*GST-S1 away from the other more abundant GST members in *F. hepatica* cytosol. This paper reports a pipeline for the purification of native *Fh*GSTS1 using a two-step process consisting of glutathione-agarose affinity and cationic exchange chromatography. The methodology allows for the isolation of purified and active *Fh*GSTS1 or Sigma GSTs from other sources for analytical biochemical and immunological studies.

1. Introduction

Fasciola hepatica, is a major parasite of livestock and humans in temperate regions throughout the world. Fascioliasis negatively impacts upon the welfare and production of domestic livestock, with infected animals exhibiting marked decreases in the yields of meat, milk and wool, reduced fertility and risk of sudden early death [1]. This loss of production is a threat to global food security, with an estimated annual cost of at least $3 billion [2]. In addition, *F. hepatica* is re-emerging as a global food borne disease of human populations, with up to 17 million people infected and a further 180 million at risk [3].

Control of *F. hepatica* has been mainly achieved through the application of short-lived anthelmintic chemicals to treat both infected livestock and humans. The drug of choice for especially the acute disease is Triclabendazole (TCBZ); unique in its ability to target both pathogenic juvenile and adult *F. hepatica* [4]. However, the over reliance on TCBZ has driven the emergence and spread of drug resistant liver fluke strains, further threatening problematic disease control [5]. Moreover, the mechanisms underpinning TCBZ resistance are currently unknown, hindering the detection of resistance at an early stage.

The declining global efficacy of TCBZ is unfortunately coupled with climate change increasing disease incidence and range [6]. In the continued absence of a TCBZ replacement there is renewed drive to develop a commercially sustainable liver fluke vaccine. Many vaccine targets for *F. hepatica* have been identified and experimentally tested, most show too low protection rates for commercial development and some fail when vaccine trials are repeated [7]. A panel of the most promising liver fluke vaccine candidates include members of the soluble glutathione transferase superfamily, such as recently discovered Sigma class glutathione transferase (*Fh*GSTS1), a Hematopoietic Prostaglandin D synthase [8,9,10,11].

*Fh*GSTS1 has been shown to be secreted from liver fluke via extracellular vesicles, exhibit prostaglandin synthase activity, suppress a Th17 response in host dendritic cells via TLR-4 dependent binding and stimulate increased prostaglandin release from host cells [12,13,14]. These abilities suggest that *Fh*GSTS1 has keys establishment roles within the host and importantly the location of this protein, in the tegument, fluke secretions [15] and extracellular vesicles [12], makes it susceptible as part of a blocking antibody vaccine strategy and/or new target for chemical anthelmintics. However, these above immunological and

---

biochemical assays have only been performed using a recombinant form of this protein (rFhGSTS1) [10,13].

Unfortunately, vaccine trials with rFhGSTS1 did not significantly reduce the fluke challenge burden of infected goats, despite a reduction in lesions and necrotic tracts in the host liver tissue. Interestingly, previous experimental vaccinations with a native FhGST (nFhGST) isoenzyme mix likely incorporated FhGSTS1 protein and provided almost a 70% reduction in worm burden in a cattle trial [16]. Furthermore, it has been shown recently that the nFhGSTS1 likely undergoes O-linked glycosylation [17] and this may be an important component of a protective vaccine formulation that has not been tested by current recombinant forms.

To date, native GST studies are hampered in parasitic worms by lack of methodology to separate Sigma GST protein from other more abundant soluble GST classes such as Mu [18]. Thus, the aim of this investigation was to isolate nFhGSTS1 from somatic extracts of adult F. hepatica through a two-step purification pipeline utilising GSH-agarose affinity chromatography, followed by cationic exchange chromatography in order to exploit the relatively high pI of FhGSTS1. Finally, nFhGSTS1 enrichment and purification were confirmed using 2DE, western blotting and tandem mass spectrometry.

2. Materials and methods

2.1. Protein sample preparation

Adult liver fluke were obtained from freshly condemned sheep livers at a local abattoir in West Wales and were extensively washed in PBS then stored at $-80\,^{\circ}C$ until required. Somatic protein was extracted on ice from 5 g of pooled adult F. hepatica in 1:10 (v/v) homogenisation buffer (10 mM sodium phosphate, 1% v/v Triton X-100, pH 7.4, 1 tablet of cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich)) and centrifuged at $21,000 \times g$ for 30 min at $4\,^{\circ}C$, with the supernatant containing soluble proteins, including GST classes, collected, sonicated for three 15 s pulses and termed the somatic extract.

2.2. Protein concentration and GST enzymatic assay

Protein concentration was estimated using the method of Bradford [19]. The CDNB (1-Chloro-2,4-dinitrobenzene) glutathione conjugation model substrate assay was used to measure GST activity [20]. The assay was completed in a 1 ml volume, with a final concentration of 100 mM potassium phosphate buffer (pH 6.5), 1 mM reduced glutathione, 1 mM CDNB and absorbance changed measured at 340 nm for 3 min at $20\,^{\circ}C$. The model substrate ethacrynic acid was also tested according to the method previously described [13].

2.3. Glutathione-agarose affinity chromatography

A 1 ml volume matrix of GSH-agarose was prepared in column format according to the manufactures instructions (Sigma Ltd.). In brief, the matrix was equilibrated with 20 column volumes (CVs) of 10 mM sodium phosphate buffer pH 7.4 containing 150 mM NaCl at a flow rate of 1.5 ml/min at room temperature. Somatic extract was passed through the column under gravity flow a total of six times. The column was washed with 20 CVs of the previously described equilibration buffer. The elution of bound protein was achieved by passing 5 CVs of elution buffer (50 mM Tris–HCl) pH 8.0 containing 5 mM GSH, at a flow rate of 1.0 ml/min at room temperature. The collected eluent was then concentrated using 10 kDa MWCO centrifuge filter units (Amicon) and exchanged to 20 mM sodium phosphate buffer, pH 6.8.

2.4. Cationic exchange chromatography

Pre-packed 1 ml, HiTrap, fast-flow, carboxy-methyl (CM) Sepharose columns from G. E. Healthcare Ltd. were linked to an automated ÄKTA prime plus system (G. E. Healthcare). All buffers and solutions for CM chromatography were passed through 0.45 μm cellulose filters. Initially, the system was washed and primed with buffer A (20 mM sodium phosphate buffer, pH 6.8) with the column installed with a system flow rate of 1.0 ml/min. Equilibration of the column was achieved by washing through 20 CVs of buffer A. The GSH-affinity purified sample was loaded into the AKTA system at $4\,^{\circ}C$ using a 500 μl sample loop and passed through the column at the optimal flow rate of 1.0 ml/min. The column was washed again with 20 CVs of buffer A. Adsorbed protein was eluted from the column using a single step increase in NaCl concentration achieved by passing 100% buffer B (20 mM sodium phosphate, 1.0 M NaCl, pH 6.8) through the column. PrimeView version 1.0 software (G. E. Healthcare) was used to monitor protein flow through, at 220 nm. Protein peaks in the UV chromatograph were collected in 1 ml fractions and protein containing fractions pooled for subsequent analyses.

2.5. 1-Dimensional SDS-PAGE

Protein profiles were resolved by 1D SDS-PAGE. Samples were denatured in Laemmli sample buffer (BioRad) and heated to $95\,^{\circ}C$ for 15 min and separated on a 12.5% acrylamide gel using a mini-Protean kit (Biorad) equilibrated with TGS gel running buffer (25 mM Tris, 192 mM glycine, 0.1% w/v SDS, pH 8.6). The protein separation was completed at 70 V for 30 min and then 150 V for 90 min. Proteins were visualised using colloidal Coomassie staining [20] and imaged using a GS-800 densitometer (Biorad) and analysed with QuantityOne software (Biorad).

2.6. 2-Dimensional electrophoresis

Isoelectric focusing was carried out using 7 cm linear pH 3–10 immobilized Readystrip™ IPG strips (BioRad). In brief, the IPG strips were rehydrated for 16 h with a solution comprising of the protein sample and buffer Z (8 M urea, 2% w/v CHAPS, 33 mM DTT, 0.5% v/v ampholytes pH 3–10). The hydrated strips were focused using a Protean Kit (Biorad) for 10,000 VH at $20\,^{\circ}C$. After focusing the IPG strips were placed in equilibration buffer (50 mM Tris–HCl, 6 M urea, 30% v/v glycerol, 2% w/v SDS, pH 6.8) containing 10 mg/ml DTT, for 15 min at room temperature. A second equilibration step followed in which the IPG strip was placed in same equilibration buffer containing 25 mg/ml IAA. After equilibration the IPG was placed on to a 12.5% acrylamide gel and fixed in position with molten agarose. Separation by molecular weight was performed according to the conditions for 1D SDS-PAGE.

2.7. Western blotting

Western blotting was performed using a mini blot kit (Biorad), with the protein transferred from 1D SDS-PAGE 12.5% acrylamide gels to nitrocellulose membrane. Prior to blotting, the gels and membranes were soaked in western blot equilibration buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 20 min. Transfer of protein to membrane was performed over a two hour period at 40 V. Transfer was confirmed by amido black staining (0.1% w/v amido black, 45% v/v methanol, 10% v/v acetic acid) for 1 min. The membrane with transferred protein was subject to three 10 min washes in Tween-tris-buffered saline (TTBS; 0.1 M Tris, 1% v/v Tween, 0.9% w/v NaCl, pH 7.5) and blocked in TTBS with 5% w/v powdered skimmed milk, overnight at $4\,^{\circ}C$.

Immuno-detection of Mu and Sigma class FhGSTs followed the protocol of Morphew et al. [21]. Briefly, the blotted nitrocellulose membrane was blocked in TTBS with 1% w/v skimmed milk powder. For the detection of Mu GST classes, anti-Mu Schistosoma japonicum polyclonal antibodies (Sigma-Aldrich) raised in rabbit were used as parasitic flatworm Mu class representatives at a concentration of 1: 5000 (v/v) and for Sigma GST class anti-FhGSTS1 polyclonal antibodies

raised in rabbits were used at a concentration of 1:30,000 (v/v). The membranes were incubated with primary antibodies for 60 min at room temperature. Following three washes in TBS, the membranes were incubated in TTBS with anti-goat and anti-rabbit IgG alkaline phosphatase secondary antibodies (Sigma Ltd) at a concentration of 1: 30,000 for 60 min at room temperature. Visualisation of antibody binding was developed using the BCIP/NBT system [22].

2.8. Protein identification by mass spectrometry

Protein spots were excised from the 2D SDS-PAGE gel and destained in three washes of 50 mM ammonium bicarbonate (pH 8) and acetonitrile, at a 1:1 ratio, at 37 °C. The gel pieces were dehydrated in acetonitrile for 15 min at 37 °C followed by air drying at 50 °C until all the liquid was removed. The gel pieces were swollen with 50 mM ammonium bicarbonate containing sequencing grade modified trypsin (Promega), at a concentration of 10 µg/µl, and incubated at 37 °C overnight. 20 µl of double distilled water was added and the samples agitated for 10 min and the supernatant was collected. To the gel piece, 50 µl of 50% (v/v) 50 mM ammonium bicarbonate (pH 8), 5% (v/v) formic acid was added and agitated for a further 60 min after which the supernatant was collected and mixed with the previously collected supernatant. The mix was dried using a speed vacuum and stored at −20 °C until required. The samples were reconstituted in 20 µl of 1% (v/v) formic acid and centrifuged at $3000 \times g$ for 3 min to remove particulates, with 15 µl of the supernatant collected and placed in a sterile tube.

Protein spot samples were analysed using liquid Chromatography tandem mass spectrometry (Agilent 6550 iFunnel Q-TOF) coupled to a HPLC-Chip (1200 series, Agilent Technologies, Cheshire, UK). The HPLC-Chip/Q-TOF system was equipped with a capillary loading pump (1200 series, Agilent Technologies) and a nano pump (1200 series, Agilent Technologies). Sample injection was conducted with a micro auto sampler (1100 series, Agilent Technologies), where 1 µl of sample in 0.1% v/v formic acid was loaded on to the enrichment column at a flow of 2.5 µl/min followed by separation at a flow of 300 nl/min. A Polaris Chip was used (G4240-62030, Agilent Technologies), comprising a C18 enrichment/trap column (360 nl) and a C18 separation column (150 mm × 75 Âµm), where ions were generated at a capillary voltage of 1950 V. The solvent system was: solvent A (ultra-pure water with 0.1% v/v formic acid), and solvent B (90% v/v acetonitrile with 0.1% v/v formic acid). The liquid chromatography was performed with a piece-linear gradient using 3–8% of solvent B over 0.1 min, 8–35% solvent B over 14.9 min, 35–90% solvent B over 5 min and hold at 90% solvent B for 2 min. Tandem mass spectrometry was performed in AutoMS2 mode in the 300–1700 Da range, at a rate of 5 spectra per second, performing MS2 on the 5 most intense ions in the precursor scan. Masses were excluded for 0.1 min after MS/MS was performed. Reference mass locking was used for internal calibration using the mass of 391.2843 Da.

The data was filtered by molecular feature and exported as a MASCOT generic file (MGF). Using a local 'in house' MASCOT database (www.matrixscience.com) of the *F. hepatica* genome, the acquired MGF files submitted for a MS/MS ion search, using parameters previously described [21]. Homology was determined to be significant ($p < 0.05$) for proteins with MASCOT ion scores greater than 32. The transcript identities were then searched at WormBase ParaSite (https://parasite.wormbase.org/index.html) in order to obtain the primary sequence of the protein. Protein identity was then confirmed by submitting the acquired primary sequence to blastp (www.ncbi.nlm.nih.gov) search against non-redundant protein sequences.

3. Results

5 g of adult *F. hepatica* worms yielded 105 mg of soluble somatic protein with a total CDNB glutathione conjugating activity of 173,161 nmol min^{-1}, thus with a somatic GST specific activity of

Table 1
Purification of *F. hepatica* GSTs using GSH-agarose affinity chromatography and CM-Sepharose cation exchange chromatography. *Unit (U) defined as the amount of GST required to react with 1 nmol CDNB per min. Average specific activity was determined from 3 replicates, ± S.D.

| Fraction	Protein (mg)	Specific activity (U*/mg)	Total activity (U)
Somatic extract	104.9	1651.0 ± 96.5	173,161.1
GSH-affinity	4.1	13,866.4 ± 906.6	56,436.1
CM-Sepharose	0.05	1471.5 ± 345.5	73.6

1651 ± 96.5 nmol min^{-1} mg^{-1} protein. The first stage of the purification separated the GST isoenzyme mix from the somatic extract protein using GSH-affinity chromatography. The step removed approximately 96% of the somatic protein and the isolated proteins had 8.4 fold enrichment in GST specific activity (13,866 ± 906.6 nmol min^{-1} mg^{-1} protein), which accounted for 32.6% of the starting CDNB glutathione conjugating activity present in the somatic extract (Table 1).

Cationic exchange (CM-Sepharose) was used to isolate basic p*I* n*Fh*GSTS1 away from other acidic and near neutral p*I* GSH-agarose binding GSTs. This second stage required optimisation of the initial pH conditions i.e. adsorption of n*Fh*GSTS1 and the removal of the non-interacting Mu class *Fh*GSTs. n*Fh*GSTS1 was predicted to have a p*I of* 8.86 using the compute pI/MW tool (http://www.expasy.org/proteomics) and this was similar to the published experimental value of 8.56 [11]. Thus, purifications were carried out with various equilibration buffer pH ranges and the optimal conditions determined to be at pH 6.8. Under the optimised conditions, a single elution peak was observed in the UV chromatogram (Fig. 1A) when the column was washed with 100% buffer B. Due to the low concentration of eluted protein, fractions 44 and 45 were pooled and determined to have a specific activity of 1471 ± 345.5 nmol min^{-1} mg^{-1} protein (Table 1). The CM-Sepharose purified sample produced a single band on 1D SDS-PAGE with an approximate molecular weight of 25 KDa (Fig. 1B). Although classes of *Fh*GSTs were not identified at this stage when separated by reduced 1D SDS-PAGE Mr, a single protein band of the CM-Sepharose eluent was noted when compared to the doublet protein bands produced from the GSH-affinity purified and CM-Sepharose unbound fractions (Fig. 1B).

The eluted fraction of the CM-Sepharose purification produced a significant signal when subjected to western blotting with an anti-*Fh*GSTS1 polyclonal antibody and developed using the NBT/BCIP method (Fig. 2). Importantly, the same fraction did not cross-react with an anti-Mu class GST antibody. The unbound flow through fractions of the CM-Sepharose column were recognised by both anti-*Fh*GSTS1 antibodies, albeit weakly, and with anti-Mu class GST antibodies (Fig. 2).

For further integrity, the CM-Sepharose eluted fractions were separated by 2D SDS-PAGE and resolved to a single protein spot with an estimated p*I* of 8.17 (Fig. 3).

Analysis using MS/MS spectroscopy revealed that this protein spot consisted of a single protein which when matched to an 'in house' *F. hepatica* MASCOT database was confirmed to be *Fh*GSTS1 (MASCOT Score 669; Accession no. 2WB9_A; Fig. 4). Identity was confirmed through the sequencing of 33 peptides relating to 7 distinct peptide sequences and providing sequence coverage of 33%. The full protein identification results can be found in Supplementary Table 1. Importantly, no other protein spots were detected by the colloidal Coomassie stain showing that n*Fh*GSTS1 is the only protein present in the sample and it remains stable throughout this method of purification (Fig. 3). Furthermore, n*Fh*GSTS1 retained activity towards CDNB (Table 1) and also exhibited activity towards the model substrate ethacrynic acid with an average specific activity of 1482.25 nmol min^{-1} mg^{-1} protein (n = 2), this was higher than the specific activity measured for r*Fh*GSTS1 [13].
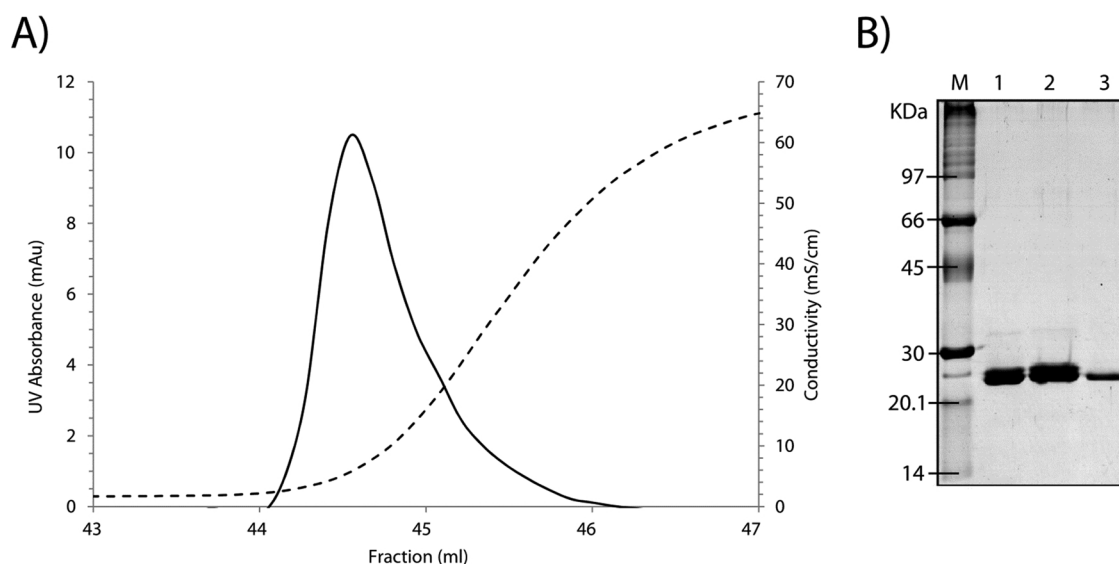
Fig. 1. Purification of FhGSTS1. A) The elution profile of protein bound to CM-Sepharose equilibrated to pH 6.8. Removal of protein from the column was achieved through a single step increase in NaCl concentration, shown by the increase in conductivity (dashed line). B) 1D SDS-PAGE gel stained with colloidal Coomassie showing different stages of purification, 1) GSH-affinity purified protein, 2) Cationic exchange unbound flow through protein, 3) CM-Sepharose purified protein.

4. Discussion

This investigation has delivered a new pipeline for the production of purified n*Fh*GSTS1 from somatic protein extracts of adult *F. hepatica* tissue. Importantly, the end-product of this two-step process was a structurally stable and enzymatically active native protein, supporting robust immunological and biochemical studies.

Interestingly, n*Fh*GSTS1 exhibited greater ethacrynic acid glutathione conjugation activity and less CDNB glutathione conjugation activity than r*Fh*GSTS1 [13]. The differences in substrate activities between the recombinant and the native form of *Fh*GSTS1 further highlight the potential limitation of bacterial host protein production technology and lack of eukaryotic post-translational modifications may have had consequence on the previous r*Fh*GSTS1 vaccine studies. Thus, native proteins may be required to fully understand host-parasite interactions in order to realise liver fluke protective vaccines [24]. However, with respect to future research, native parasitic worm protein isolation strategies is challenging as it requires removing parasites from vertebrates with often relatively low yield of final purified antigen. In this study each batch of purification round required 5 g of *F. hepatica* tissue to yield 50 μg of purified target protein to study.

Previous proteomic work identified Sigma GST protein in more near neutral protein spots (p*I* 7.1) along with Mu class GSTs in *F. hepatica* extracts [11]. This finding is further supported in this biochemical study as Sigma GST was also detected in the CM-Sepharose unbound fractions, suggesting that a more acidic modified form of n*Fh*GSTS1 or an additional Sigma class GST may also be expressed. Native GSTs consist of two subunits, either as homodimers or heterodimers [20]. Thus, the formation of native heterodimeric *Fh*GSTs with extended functions in
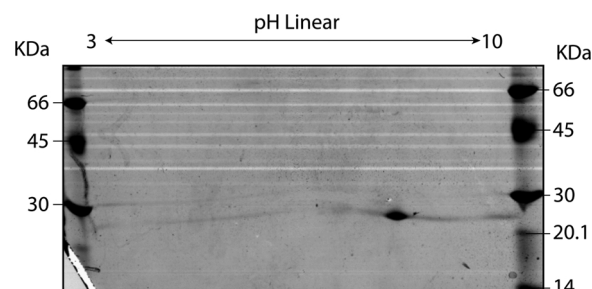

Fig. 3. Confirmation of *n*FhGST-S1 purity with 2DE. 1 μg of GSH-affinity and CM-Sepharose purified sample were separated by 12.5% polyacrylamide/p*I* 3–10 and visualised using colloidal Coomassie stain. Tandem mass spectrometry identified the solitary protein spot as *Fh*GSTS1 (Fig. 4).

liver fluke cannot be also be ruled out, especially as heterodimeric GSTs have recently been described in another parasitic flatworm, the cestode *Echinococcus granulosus* [23].

In conclusion, our new methodology allows for the isolation of purified and active *Fh*GSTS1 or basic p*I* Sigma GSTs other sources for analytical biochemical and immunological studies, such as vaccination trials in rodent models. We envisage that the column bed volumes can be increased for more preparative studies.

Acknowledgement
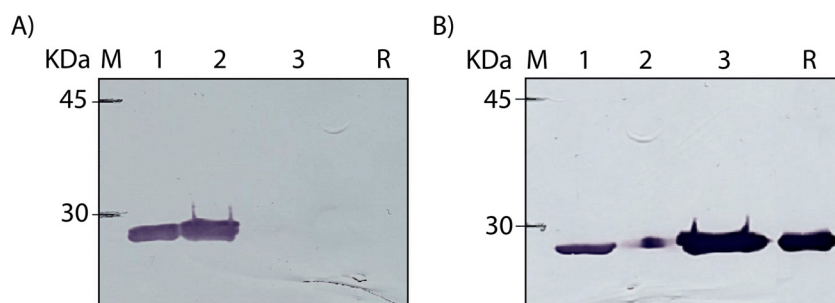
Fig. 2. Western blot analysis of the different stages of *n*FhGSTS1 purification, developed using NBT/BCIP system. A total of 2 μg of protein was loaded onto each lane.1) GSH-affinity purified GSTs 2) CM-Sepharose unbound flow through 3) CM-Sepharose purified R) recombinant *Fh*GSTS1 control. A) Membrane probed with anti-mu class antibodies (1:5000 dilution). B) Membrane probed with anti-sigma class antibodies (1:30,000).

A) MDKQHFKLWYFQFRGRAEPIR|**LLLTCAGVK**|FEDYQFTMDQWPTIKPTLPGG
R|**VPLLDVTGPDGK**|LRR|**YQESMAIAR**|LLARQFK|**MMGETDEEYYLIER**|**I
IGECEDLYR**|EVYTIFRTPQGEKEAKIK|**EFKENNGPTLLK**|**LVSESLESSGG
K**|HVAGNRITLGDLFLFTTLTHVMETVPGFLEQKFPKLHEFHKSLPTSCSRLS
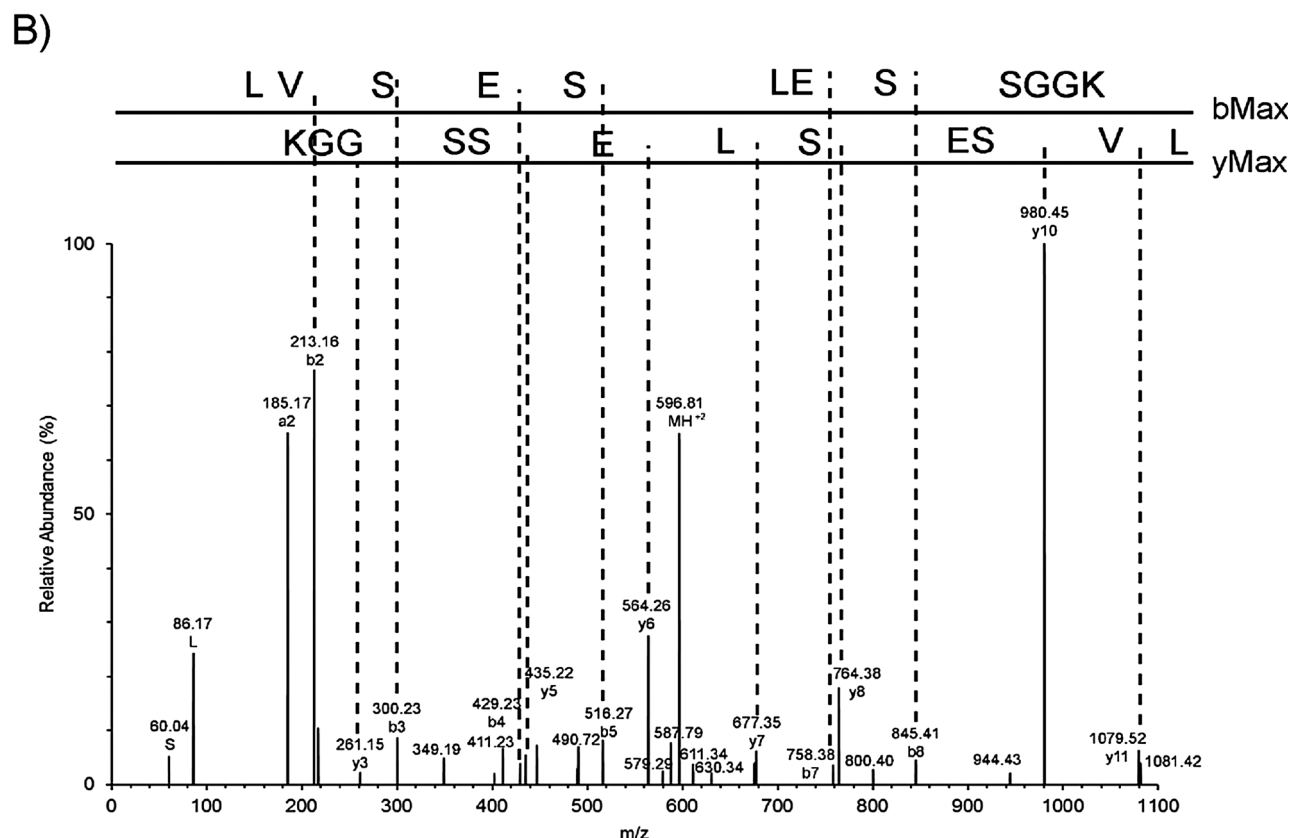EYLKKRAKTPF

B)

Fig. 4. Confirmation of *n*FhGSTS1 via MSMS. A) Protein sequence of *n*FhGSTS1 accession no. 2WB9_A. Highlighted in bold are the seven identified peptides during MSMS including the characteristic peptide of this GST (bold and underlined) which is highlighted in B) Annotated tandem mass spectra from the analysis of the protein spot (Fig. 3) confirming the identity of the purified protein to be *n*FhGSTS1.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.molbiopara.2018.04.006.

References

[1] D. PIedrafita, T.W. Spithill, R.E. Smith, H.W. Raadsma, Improving animal and human health through understanding liver fluke immunology, Parasite Immunol. 32 (8) (2010) 572–581, http://dx.doi.org/10.1111/j.1365-3024.2010.01223.x.

[2] J.C. Boray, Disease of Domestic Animals Caused by Flukes, Food and Agricultural Organization of the United Nations, Rome, 1994 49 pp..

[3] M. Nyindo, A.H. Lukambagire, Fascioliasis: an ongoing zoonotic trematode infection, BioMed Res. Int. 2015 (2015) 8, http://dx.doi.org/10.1155/2015/786195 Article ID 786195.

[4] J. Boray, P. Crowfoot, M. Strong, J. Allison, M. Schellenbaum, M. Von Orelli, G. Sarasin, Treatment of immature and mature *Fasciola hepatica* infections in sheep with triclabendazole, Vet. Rec. 113 (14) (1983) 315–317, http://dx.doi.org/10.1136/vr.113.14.315.

[5] J.M. Kelley, T.P. Elliott, T. Beddoe, G. Anderson, P. Skuce, T.W. Spithill, Current threat of triclabendazole resistance in *Fasciola hepatica*, Trends Parasitol. 32 (6) (2016) 458–469.

[6] N.J. Fox, P.C. White, C.J. McClean, G. Marion, A. Evans, M.R. Hutchings, Predicting impacts of climate change on *Fasciola hepatica* risk, PLoS one 6 (1) (2011) e16126.

[7] H. Toet, D. PIedrafita, T. SpIthill, Liver fluke vaccines in ruminants: strategies, progress and future opportunities, Int. J. Parasitol. 44 (12) (2014) 915–927, http://dx.doi.org/10.1016/j.ijpara.2014.07.011.

[8] O. Golden, R.J. Flynn, C. Read, M. Sekiya, S.M. Donnelly, C. Stack, J.P. Dalton, G. Mulcahy, Protection of cattle against a natural infection of *Fasciola hepatica* by vaccination with recombinant cathepsin L1 (r*Fh*CL1), Vaccine 28 (34) (2010) 5551–5557, http://dx.doi.org/10.1016/j.vaccine.2010.06.039.

[9] P.A. Nambi, S.C. Yadav, O.K. Raina, D. Sriveny, M. Saini, Vaccination of buffaloes with *Fasciola gigantica* recombinant fatty acid binding protein, Parasitol. Res. 97 (2) (2005) 129–135, http://dx.doi.org/10.1007/s00436-005-1397-4.

[10] R. Zafra, R.A. Pérez-Écija, L. Buffoni, I.L. Pacheco, A. Martínez-Moreno, E.J. LaCourse, S. Perally, P.M. Brophy, J. Pérez, Early hepatic and peritoneal changes and immune response in goats vaccinated with a recombinant glutathione transferase sigma class and challenged with *Fasciola hepatica*, Res. Vet. Sci. 94 (3) (2013) 602–609.

[11] G. Chemale, R. Morphew, J.V. Moxon, A.L. Morassuti, E.J. LaCourse, J. Barrett, D.A. Johnston, P.M. Brophy, Proteomic analysis of glutathione transferases from the liver fluke parasite, *Fasciola hepatica*, Proteomics 6 (23) (2006) 6263–6273, http://dx.doi.org/10.1002/pmic.200600499.

[12] K. Cwiklinski, E. de la Torre-Escudero, M. Trelis, D. Bernal, P.J. Dufresne, G.P. Brennan, S. O'Neill, J. Tort, S. Paterson, A. Marcilla, J.P. Dalton, The extracellular vesicles of the helminth pathogen, *Fasciola hepatica*: biogenesis pathways and cargo molecules involved in parasite pathogenesis, Mol. Cell. Proteomics 14 (12) (2015) 3258–3273, http://dx.doi.org/10.1074/mcp.M115.053934.

[13] E.J. LaCourse, S. Perally, R.M. Morphew, J.V. Moxon, M. Prescott, D.J. Dowling, S.M. O'Neill, A. Kipar, U. Hetzel, E. Hoey, R. Zafra, P.M. Brophy, The Sigma class glutathione transferase from the liver fluke *Fasciola hepatica*, PLoS Negl. Trop. Dis. 6 (5) (2012) e1666.

[14] D.J. Dowling, C. Hamilton, S. Donnelly, E.J. La Course, P.M. Brophy, J. Dalton, S.M. O'Neill, Major secretory antigens of the helminth *Fasciola hepatica* activate a suppressive dendritic cell phenotype that attenuates Th17 cells but fails to activate Th2 immune responses, Infect. Immun. 78 (2) (2009) 793–801.

[15] J. Creaney, G. Wijffels, J. Sexton, R. Sandeman, T. Spithill, J. Parsons, *Fasciola hepatica*: localization of glutathione S-transferase isoenzymes in adult and juvenile liver fluke, Exp. Parasitol. 81 (1) (1995) 106–116.

[16] C.A. Morrison, T. Colin, J.L. Sexton, F. Bowen, J. Wicker, T. Friedel, T.W. Spithill, Protection of cattle against *Fasciola hepatica* infection by vaccination with glutathione S-transferase, Vaccine 14 (17–18) (1996) 1603–1612.

[17] A. Garcia-Campos, A. Ravidà, D.L. Nguyen, K. Cwiklinski, J.P. Dalton, C.H. Hokke, S. O'Neill, G. Mulcahy, Tegument glycoproteins and cathepsins of Newly Excysted Juvenile *Fasciola hepatica* carry mannosidic and paucimannosidic N-glycans, PLoS Negl. Trop. Dis. 10 (5) (2016) e0004688, http://dx.doi.org/10.1371/journal.pntd.0004688.

[18] P.M. Brophy, P. Crowley, J. Barrett, Detoxification reactions of *Fasciola hepatica* cytosolic glutathione transferases, Mol. Biochem. Parasitol. 39 (2) (1990) 155–161.

[19] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1–2) (1976) 248–254.

[20] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione S-transferases the first enzymatic step in mercapturic acid formation, J. Biol. Chem. 249 (22) (1974) 7130–7139.

[21] R.M. Morphew, N. Eccleston, T.J. Wilkinson, J. McGarry, S. Perally, M. Prescott, D. Ward, D. Williams, S. Paterson, M. Raman, G. Ravikumar, P.M. Brophy, E.J. LaCourse, Proteomics and in silico approaches to extend understanding of the glutathione transferase superfamily of the tropical liver fluke *Fasciola gigantica*, J. Proteome Res. 11 (12) (2012) 5876–5889, http://dx.doi.org/10.1021/pr300654w.

[22] M.S. Blake, G.J. Russell-Jones, K.H. Johnston, E.C. Gotschlich, A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots, Anal. Biochem. 136 (1) (1984) 175–179, http://dx.doi.org/10.1016/0003-2697(84)90320-8.

[23] P. Arbildi, S. La-Rocca, V. Lopez, N. Da-Costa, V. Fernandez, *Echinococcus granulosus*: evidence of a heterodimeric glutathione transferase built up by phylogenetically distant subunits, Mol. Biochem. Parasitol. 211 (2017) 26–30.

[24] V. Molina-Hernández, G. Mulcahy, J. Pérez, Á. Martínez-Moreno, S. Donnelly, S.M. O'Neill, J.P. Dalton, K. Cwiklinski, *Fasciola hepatica* vaccine: we may not be there yet but we're on the right road, Vet. Parasitol. 208 (1) (2015) 101–111, http://dx.doi.org/10.1016/j.vetpar.2015.01.004.